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Reversible Modification of Thiol-Containing Polypeptides with Poly(ethylene glycol) Through Formation of Mixed Disulfide Bonds

The Case of Papaya Proteinase III

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ABSTRACT

Papaya proteinase III (PPIII) was purified, as the S-methylthio derivative from the latex of *Carica papaya L.*, by ion-exchange chromatography. Separation of reactivable PPIII from the irreversibly oxidized molecular species of this enzyme was readily achieved after a selective conversion of the reactivated proteinase into the S-monomethoxypoly-(ethylene glycol)thio derivative (S-mPEG thio PPIII). From this derivative, a PPIII preparation titrating 1 mol of thiol/mol of enzyme was regenerated. From the physicochemical properties of S-mPEG thio PPIII that were investigated, it is concluded that interactions between the mPEG and the PPIII chains occur only to a limited extent. In addition to its usefulness for purifying thiol-containing enzymes, the mPEG modification resulting from mixed disulfide bond formation may find other practical applications.

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Index Entries: mPEG; covalent attachment; cysteine-proteinase; covalent chromatography; affinity chromatography.

INTRODUCTION

The potential value of peptides and proteins as therapeutics has been recognized for a long time; unfortunately, their delivery gives rise to a series of problems with regard to their low bioavailability. Entrapment into liposomes or nanoparticles has been shown to increase the therapeutic efficiency of peptide drugs.

In addition to these techniques, the covalent attachment of poly(ethylene glycol) (PEG) chains to polypeptides has also recently been reported to be successful. This technique confers to the modified polypeptides the additional and interesting properties of the PEG chains themselves (*1*).

As a general rule, PEG-modified polypeptides exhibit increased stability, resistance to proteolytic inactivation, and consequently increased circulating lives. Pegylation also decreases antigenicity and immunogenicity (*2,3*).

Prolonged therapeutic efficiency is observed when making use of stable conjugates that are resistant to chemical attacks (e.g., hydrolysis) under conditions similar to the physiological ones. Irreversible modifications of polypeptides by PEG derivatives have thus been searched for.

Several chemical procedures are presently available to achieve this aim. For instance, amino functions of lysyl side chains have been modified through formation of carbamate (*4,5*) or amide (*6*) linkages. Attachment of PEG to the guanidino function of arginyl residues was performed with PEG analogs of phenylglyoxal (*7*) and to the thiol function of cysteinyl side chains with PEG-maleimides (*8*). Only two exceptions were reported where reversibility of the modification by PEG was intentionally selected.

Garman (*9*) patented the synthesis of a PEG analog of dimethylmaleic anhydride able to attach PEG to amino groups of polypeptides through an amide linkage that was slowly hydrolyzed under physiological conditions. The validity of this approach to generate active proteins was demonstrated with plasminogen activator. The PEG–plasminogen activator conjugates obtained by this method yielded active and completely deacylated protein after 44 h of incubation at pH 7.4 and 37°C (*9*).

On the other hand, Glass and coworkers (*10*) reported the preparation of 4-phenoxy-3,5-dinitrobenzoyl-PEG. This PEG derivative reacts rapidly and selectively at neutral pH with sulfhydryl groups of peptides to yield polymer–peptide adducts in which the components are linked to a thiol-sensitive dinitrophenylene. It was thus possible to attach the thiol-containing peptide to the polymer and, after performing some chemical and/or enzymatic alterations on the conjugate, to remove the modified peptide from the PEG carrier (*10*).

It is therefore expected that the reversible modification of polypeptides by PEG may provide some improvements. It may be anticipated that one should be able to take advantage of the properties that PEG confers to the conjugate while preserving the possibility to remove the PEG chain(s) when these properties are no more required.

Several possibilities exist to modify reversibly the amino, the guanidino, and/or the thiol functions of polypeptides. According to our experience, we expect that a better control should be exerted by using thiol functions. We therefore synthesized a monomethoxy PEG (mPEG) derivative selected to react specifically and instantaneously with free thiol functions of polypeptides by forming mixed disulfide bonds. This mPEG derivative called here mPEG-SS-Py is itself a mixed disulfide between a PEG derivative containing a free thiol and 2-thiolpyridine. It offers the advantage, when reacting with a thiol-containing polypeptide, to liberate 2-thiopyridone, which can be quantitatively determined spectrophotometrically at 343 nm (*11*). The usefulness of the reversible modification by mPEG-SS-Py was evaluated using PPIII, a monothiol cysteine-proteinase, which belongs to the papain superfamily, obtained by purification of the latex of *Carica papaya L.* (*12*) and for which the amino acid sequence (*13*), the 3-D crystallographic structure (*14*), and the specificity requirements (*15*) are indeed well documented.

MATERIALS AND METHODS

Aldrich-Chemie (D-7429, Steinheim, Germany) provided mPEG (mol wt 5 kDa), cystamine dihydrochloride, *N*-hydroxysuccinimide, reduced glutathione, cysteamine hydrochloride, dithiothreitol (DTT), fluorescamine, 2,2′-dipyridyldisulfide, and methylmethanethiolsulfonate. L-cysteine, *N*-acetyl-L-tryptophan ethyl ester, *N*-α-benzoyl-DL-(and L)-arginine-*p*-nitroanilide (BAPNA), and bovine serum albumin were purchased from Sigma Chemical Co. (B-2880, Bornem, Germany). CM-Sephadex C-50 was obtained from Pharmacia Biotech AB (S-75182, Uppsala, Sweden). Fluka Chemie AG (CH-9470, Buchs, UK) provided phosgene as a 2*M* solution in toluene and Bio-Rad Laboratories S.A. (B-9810, Nazareth, Eke), the chemicals and the mol-wt protein markers used for electrophoresis. Spray-dried papaya latex was generously provided by Enzymase International S.A. (B-1050, Brussels).

Purification of PPIII from Spray-Dried Papaya Latex

Spray-dried papaya latex (2 g) was dissolved in water (10 mL) containing methylmethanethiolsulfonate (50 μL). The solution was then dialyzed against water (3 × 5 L; 20 h; room temperature) and centrifuged (35,000*g*; 30 min; 4°C). The supernatant was then submitted to ion-exchange chromatography on CM-Sephadex C-50. Columns (25 × 150 mm)

were used with gels pre-equilibrated with 50 mM NaOAc at pH 5.0 (the molarity of acetate buffers refers to the Na^+ concentration). Elution of the bound proteins was carried out with a linear gradient from 50 to 800 m*M* NaOAc buffer (pH 5.0; total volume: 2000 mL) at room temperature and using a flow rate of 60 mL/h. Fractions (15 mL) were collected and analyzed for A_{280}, amidase activity and conductivity. The last chromatographic peak fractions were pooled, concentrated, and dialyzed against water (3 × 5 L; 20 h; room temperature).

Synthesis of mPEG-SS-Py

Cysteamine was reacted with a 10-fold excess of 2,2′-dipyridyldisulfide and the resulting mixed disulfide was then added to a stoichiometric amount of mPEG-*N*-succinimide carbonate (*5*). Aminolysis was allowed to proceed at room temperature for 20 h. m-PEG-SS-Py was collected by filtration after precipitation in diethyl ether and recrystallized from ethyl acetate. The overall yield was 76%.

SDS-Page

The experiments were carried out on slab gels using a mini protean II cell (Bio-Rad). The resolving gels (pH 8.8; 12% acrylamide) were run at constant voltage (200 V) and prepared according to the method of Laemmli (*16*).

The stacking gels were made of 4% polyacrylamide (pH 6.8). The upper and lower chambers contained Tris-glycine buffer (pH 8.3 with 0.1% SDS). The separation was toward the anode, and bromophenol blue was used as the tracking dye. Prior to loading on the gel, the protein solutions were diluted with a buffer (buffer composition: 0.25*M* Tris-HCl at pH 6.8; 4% SDS; 23% glycerol; and 1% bromophenol blue; dithiothreitol was omitted), and the mixture was boiled for 4 min.

The gels were stained with 0.25% Coomassie blue R250 dissolved in a washing solution containing MeOH, HOAc, and H_2O (45/10/45; v/v). This latter was also used for destaining. The mol-wt markers used were: lysozyme (14.3 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), and phosphorylase B (97.4 kDa).

Fluorescence Measurements

Fluorescence was measured at 25°C with a Hitachi Perkin-Elmer model MPF-2A spectrofluorimeter equipped with an Osram XBD 150 W Xenon lamp and a RCA 1P28 photomultiplier. Excitation was performed at 285 nm, and emission spectra were scanned from 250 to 500 nm. Excitation and emission band widths were 5 nm each. The protein concentration was 4 μM. For the fluorimetric titrations, an aqueous stock solution of enzyme (0.1 m*M*) was diluted in prethermostatized buffer just before recording the emission spectrum. The buffer contained acetate, phosphate,

and borate (50 m*M* each) and Na^+ (provided by the NaOH used to adjust pH values).

Circular Dichroism Measurements

Circular Dichroism (CD) spectra were recorded on a Cary 61 spectropolarimeter. Measurements in the near-UV range (250–325 nm) were performed by using 16 μM proteinase solutions in a 2-cm cell at 20°C. CD values were reported as mean residue ellipticities and were calculated by using the relative molecular weight of 108/amino acid residue, a value deduced from the amino acid sequence work (*13*).

Infrared Spectroscopy

Infrared (IR) spectra were recorded with a Perkin-Elmer Infrared Spectrometer 1720 X equipped with a Perkin-Elmer microspecular reflectance accessory (ref. P.E. 221-0357) (*17*). The internal reflection element was a Germanium ATR plate (50 × 20 × 2 mm, Harrick EJ 2121) with an aperture angle of 45° yielding about 25 internal reflections. The spectrophotometer was continuously purged with air dried on silica column (5 × 130 cm) at a flow rate of 7 $L \cdot min^{-1}$. Spectra were recorded with a nominal resolution of 2 cm^{-1} and transferred, after scanning, to the computer.

Other Analytical Methods

PPIII concentrations were determined spectrophotometrically using a $A^{1\%}_{278}$ value of 18.3 (*12*). Thiol titrations were carried out using 2,2′-dipyridyl disulfide as the titrant; the 2-thiopyridone released in the course of the titration was determined using the value $\epsilon_{343} = 7700 M^{-1} \cdot cm^{-1}$ (*11*). Amidase activities, using DL-BAPNA as the substrate, were measured as described previously (*12*). For the kinetic measurements, a Cary 118 spectrophotometer with a thermostated cell holder (37°C) continuously monitored the release of *p*-nitroaniline from L-BAPNA at 410 nm using $\Delta\epsilon = 8800 M^{-1} \cdot cm^{-1}$ (*18*). L-BAPNA (0.5 m*M*) solutions were prepared in a buffer containing EDTA (3 m*M*), phosphate, citrate, and borate (10 m*M* each) at pH 6.80. Their concentrations were determined spectrophotometrically at 315 nm using the value $\epsilon_{315} = 13{,}000 M^{-1} \cdot cm^{-1}$ (*19*). Catalytic assays were started by adding the enzyme.

The free amino functions of proteins were determined fluorimetrically after reaction with fluorescamine (*20*).

RESULTS AND DISCUSSION

Characterization of mPEG-SS-Py

The proposed structure for mPEG-SS-Py is shown in Fig. 1 together with the schematic route chosen to synthesize this mPEG derivative.

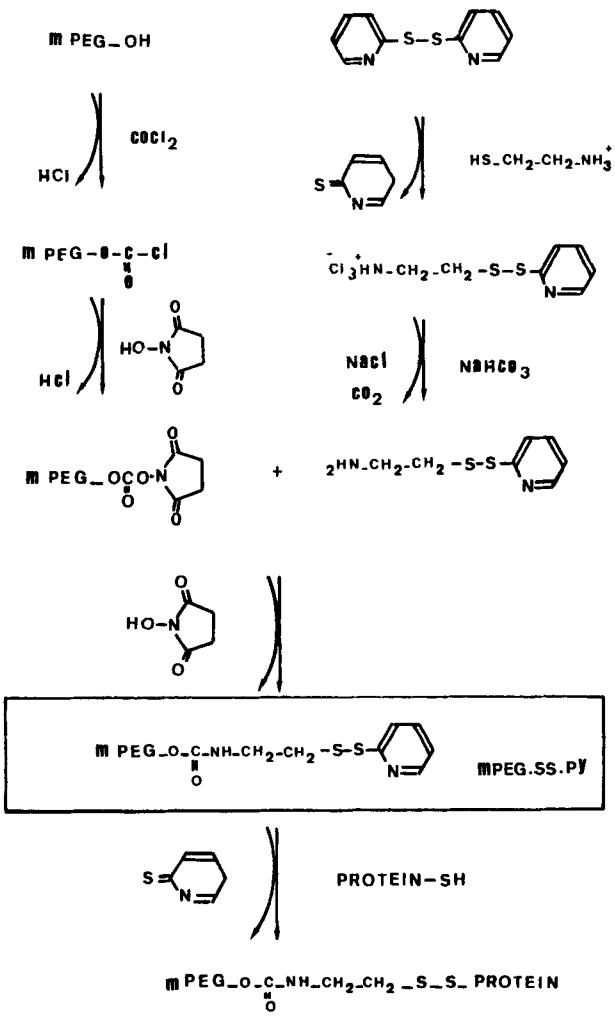

Fig. 1. Synthesis scheme of mPEG-SS-Py and of mPEG–PPIII conjugate.

Figure 1 also displays, schematically, the reaction between mPEG-SS-Py and the free thiol function of PPIII leading to the formation of a mixed disulfide bond linking mPEG and the proteinase and simultaneously to the release of 2-thiopyridone. The proposed mPEG-SS-Py structure was based on the spectral characterization as follows: mPEG-SS-Py aqueous solutions (pH 6.0; 25°C) displayed, in the near-UV range two absorption maxima at 232 (ϵ = 7120M^{-1}·cm^{-1}) and 281 (ϵ = 4580M^{-1}·cm^{-1}) nm. These spectral characteristics were very similar to those reported for *n*-propyl-2′-pyridyl disulfide (*11*). When an excess of L-cysteine was added to this mPEG-SS-Py aqueous solution, the spectral properties changed; they became identical to those of pure 2-thiopyridone and were characterized by maxima at 271 and 343 nm, respectively. Assuming that 1 mol of mPEG-

SS-Py released 1 mol of 2-thiopyridone, a value of $\epsilon_{343} = 7725 M^{-1} \cdot cm^{-1}$ is obtained, which is in excellent agreement with the value of $7700 M^{-1} \cdot cm^{-1}$ reported in the literature (*11*). These UV characteristics of mPEG-SS-Py aqueous solutions did not change after an exhaustive dialysis against water (membrane cutoff: 10 kDa). On the contrary, an aqueous solution made up of a mixture containing stoichiometric amounts of mPEG and 2,2′-dipyridyl disulfide became quite transparent in the UV range between 210 and 350 nm after dialysis. This shows that the chromophore responsible for the spectral characteristics of mPEG-SS-Py is covalently linked to the mPEG strand.

On the other hand, a mixture containing L-Lys, L-Thr, L-Arg, *S*-carboxymethyl-L-Cys, cysteic acid, and cystamine (0.1 m*M* each) does not release any 2-thiopyridone from mPEG-SS-Py (1 m*M*) after 24 h at pH 6.0 and 25°C. This experiment shows obviously that this mPEG derivative selectively reacted with thiol-containing compounds.

PREPARATION AND PURIFICATION OF THE mPEG-PPIII CONJUGATE

PPIII was purified from *Carica papaya L.* as previously described (*12*). It was recovered, as indicated by the solid bar in Fig. 2, from the last eluting proteinase fraction on the CM-Sephadex C-50 column. At this step of purification, the protein material appeared rather homogeneous by PAGE and SDS-PAGE (result not shown). Nevertheless, microheterogeneity with regard to the oxidation state of the sulfur of cysteine-25 in the active site of the enzyme was apparent.

The enzyme preparation indeed contained only 0.42 mol of reactivable thiol (2,2′dipyridyl disulfide as the titrant)/mol of enzyme. Furthermore, the specific amidase activity (DL-BAPNA as the substrate) was evaluated to 0.76 nkat/mg instead of 1.98 nkat/mg for a fully active enzyme preparation (*12*).

An important fraction of cysteine-25 in the PPIII preparation was thus irreversibly oxidized, most probably into sulfinic acid (*21,22*). On the other hand, the fraction of PPIII that contained in the starting spray-dried latex a free thiol function in position-25, was converted by us into the *S*-methylthioderivative. Still other molecular species of PPIII were probably present and were constituted by mixed disulfide forms of the proteinase with either cysteine or glutathione, found in the latex of *Carica papaya L.* Reducing microheterogeneity thus resulted from the conversion of the different molecular species of reactivable PPIII into a unique mPEG-PPIII conjugate. For that purpose, the PPIII pool (130 mg; containing 2.32 μmol of reactivable thiol) from Fig. 2 was first reactivated (15 min) in 50 m*M* NaOAc buffer, pH 5.0, in the presence of 5 m*M* dithiothreitol. Excess of

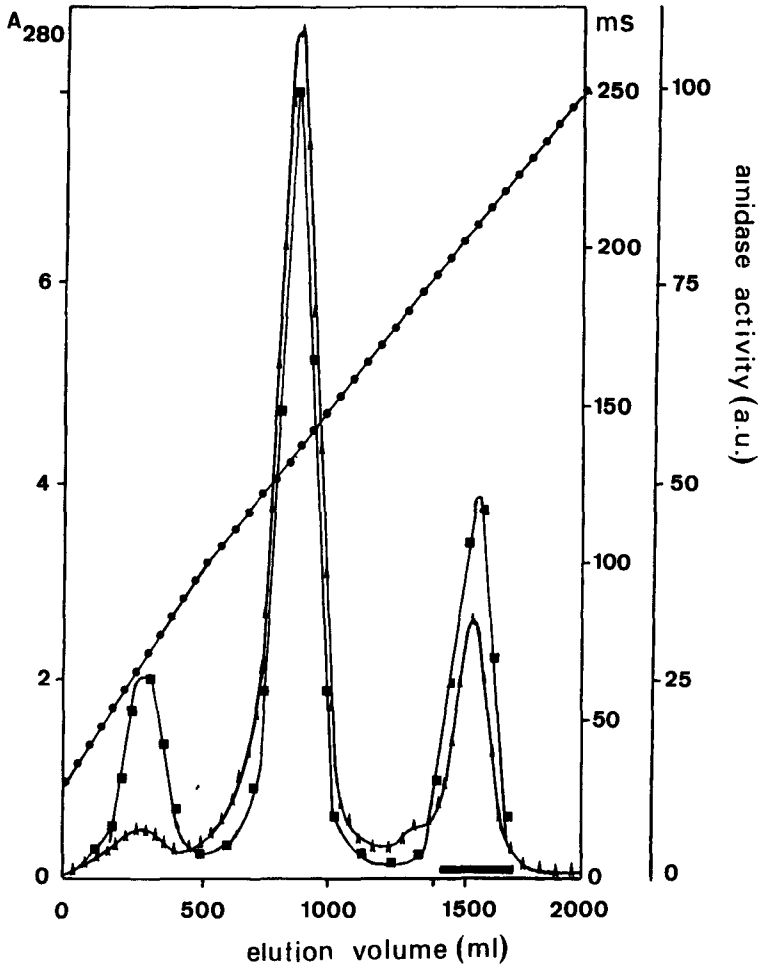

Fig. 2. Ion-exchange chromatography of the *S*-methylthioderivatives of the papaya proteinases on a column of CM-Sephadex C-50. Column: 25 × 150 mm; fractions: 15 mL; flow rate: 60 mL/h; buffer: 50 m*M* sodium acetate, pH 5.0; gradient: 50–800 m*M* Na^+, pH 5.0; total volume: 2000 mL; A_{280}: ▲—amidase activity using DL-BAPNA as the substrate: ■—conductivity (mS): ●.

dithiothreitol together with byproducts were eliminated by passage through a small CM-Sephadex C-50 column (10 × 80 mm) pre-equilibrated and eluted with the 50 m*M* NaOAc buffer. The various molecular species of PPIII were then eluted as a single fraction with 2000 m*M* of NaOAc buffer at pH 5.0 and collected in an aqueous solution containing mPEG-SS-Py (3 μmol), which resulted in the release of 2.27 μmol of 2-thiopyridone (measured spectrophotometrically at 343 nm).

The reaction mixture was then dialyzed (20 h; 4°C; against 3 × 5 L of H_2O). The mPEG-PPIII conjugate was then separated from the irreversibly oxidized forms of PPIII by ion-exchange chromatography on CM-Sephadex C-50 (*see* Fig. 3).

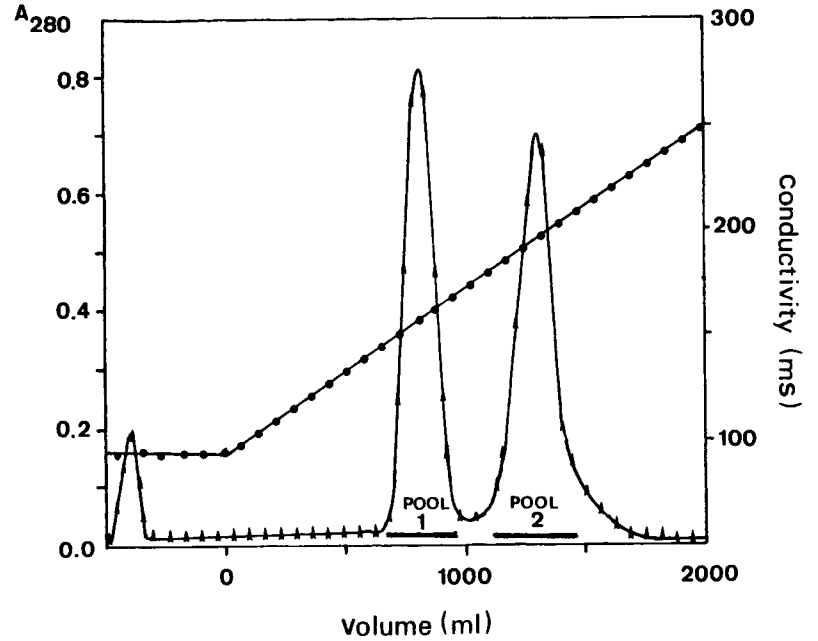

Fig. 3. Fractionation of mPEG–PPIII conjugate and oxidized PPIII on a column of CM-Sephadex C-50. Experimental conditions (except for the gradient: 200–800 m*M* Na^+) and symbols as in Fig. 2.

Preliminary Characterization of the mPEG-PPIII Conjugate

Pool 2, the last eluting chromatographic fraction from the CM-Sephadex C-50, shown in Fig. 3 contained 75 mg of protein material devoid of any detectable amidase activity toward DL-BAPNA and containing < 0.03 mol of reactivable thiol/mol of PPIII. It migrated on SDS-PAGE gels as a 24.5-kDa protein (*see* Fig. 4). On the contrary, the first eluting protein fraction from Fig. 3 (Pool 1) contained a high proportion (98.4%) of reactivable thiol and hydrolyzed DL-BAPNA with a specific activity of 2.03 nkat/mg, thus comparable to that of a fully active PPIII preparation obtained after purification by affinity chromatography (*12*).

It migrated on SDS-PAGE gels as a 32-kDa protein (Fig. 4) confirming the covalent attachment of the PEG chain. This value determined from the SDS-PAGE experiment is higher than the theoretical one calculated by summing 23.5 kDa (molecular weight of PPIII calculated from the amino acid sequence) and 5.2 kDa (molecular weight of the PEG moiety covalently attached to PPIII). This was expected since PEG standards also were reported to migrate more slowly on SDS-PAGE gels than globular protein standards with identical molecular weights (*23*). On the other hand, PPIII itself migrated more slowly than expected. This was observed by us (*12*) and by other investigators (*24*), and has been attributed to the extreme

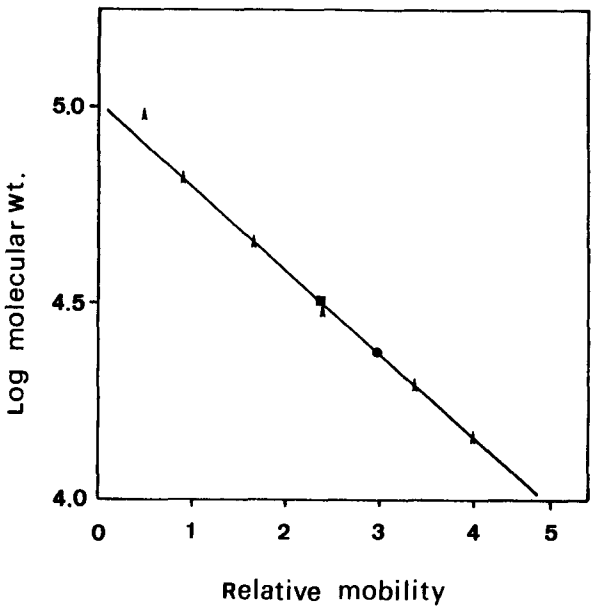

Fig. 4. Semilogarithmic plot of molecular weight vs migration distance of PPIII (●), of mPEG-PPIII conjugate (■) and of protein markers (▲). Experimental conditions in Materials and Methods.

basicity of the enzyme. An aliquot of our mPEG-PPIII conjugate was reconverted into *S*-methylthio PPIII for subsequent comparative work.

Evaluation of the Effect of the PEG Chain on the Secondary Structure and the Stability of PPIII

The observation that fully active PPIII can be regenerated from the mPEG-PPIII conjugate does not rule out the possibility that the PEG strand may modify reversibly the 3-D structure of the enzyme. This possibility was thus explored here.

The secondary structure is well conserved within the papain superfamily (*14,21,22*). Among the various elements constituting this secondary structure, helix L-1 plays a key role in the stabilization of the 3-D structure and in the modulation of the thiolate-imidazolium ion pair of these proteinases (*25*). Since the PEG chain was attached to the free thiol of cysteine-25, which is located in a part of helix L-1, it was particularly important to examine whether the presence of PEG was able to affect the secondary structure of PPIII.

Fourier Transform Infrared Spectroscopy (FTIR) was used here to determine the proportions of the different elements of the secondary structure. We used frequency ranges of 1662–1645 cm^{-1} for α-helix, 1689–1682 cm^{-1} and 1637–1613 cm^{-1} for β-sheet, 1682–1662.5 cm^{-1} for β-turns, and 1644.5–1637 cm^{-1} for aperiodic structures (*26*). The results of this study

Table 1
Compilation of the Secondary Structure Contents
for S-Methylthio PPIII and for the mPEG–PPIII Conjugate (pH 5.0)

	Percent			
Proteinase	α-Helix	β-Sheets	β-Turns	Aperiodic
S-methylthio PPIII	26.4	39.5	25.9	8.2
mPEG–PPIII conjugate	27.9	36.1	27.7	8.3

are shown in Table 1. Examination of the FTIR spectra shows that the presence of the PEG chain does not affect at all the secondary structure of PPIII. In particular, the presence of the PEG strand in the vicinity of helix L-1 does not change the α-helix content of the proteinase.

The presence of the different elements of the secondary structure and the existence of three intramolecular disulfide bonds explain why PPIII and other vegetal cysteine-proteinases are very stable enzymes that resist heat denaturation (up to 80°C) and unfolding by chaotropic agents. Nevertheless, PPIII like most other cysteine-proteinases are sensitive to acid denaturation. As shown in Fig. 5, acid denaturation of the *S*-methylthio derivative of PPIII is detectable below pH 3.0. At pH 2.0, denaturation even proceeds quite quickly. Fully denatured *S*-methylthio PPIII is devoid of any detectable amidase activity and shows typical FTIR spectra of unfolded polypeptides. Moreover, acidic denaturation of PPIII is quite irreversible. Interestingly, as shown in Fig. 5, the presence of the PEG strand in the mPEG–PPIII conjugate affords some partial protection against acid denaturation in the pH range 2.2–3.0.

Spectral Perturbations Induced by the Presence of the Covalently Linked PEG Chain

Examination of the CD spectra (*see* Fig. 6) shows that the molecular ellipticities are significantly higher for the mPEG conjugate than for the unconjugated PPIII. This observation is valid throughout the full wavelength range examined here. One may thus conclude that the presence of the PEG chain in the conjugate contributed to increase the asymmetry in the vicinity of at least some aromatic residues of PPIII.

Figure 7 displays the fluorescence emission spectra of the *S*-methylthio derivative of PPIII and of the mPEG–PPIII conjugate at pH 8.0. As compared to the former, the last one is characterized by a shift of λ_{max} toward shorter wavelengths and a drastic decrease of quantum yield (half that of the *S*-methylthio PPIII derivative) resulting in a calculated differential emission spectrum, characterized by a maximum at 350 nm (also shown in Fig. 7).

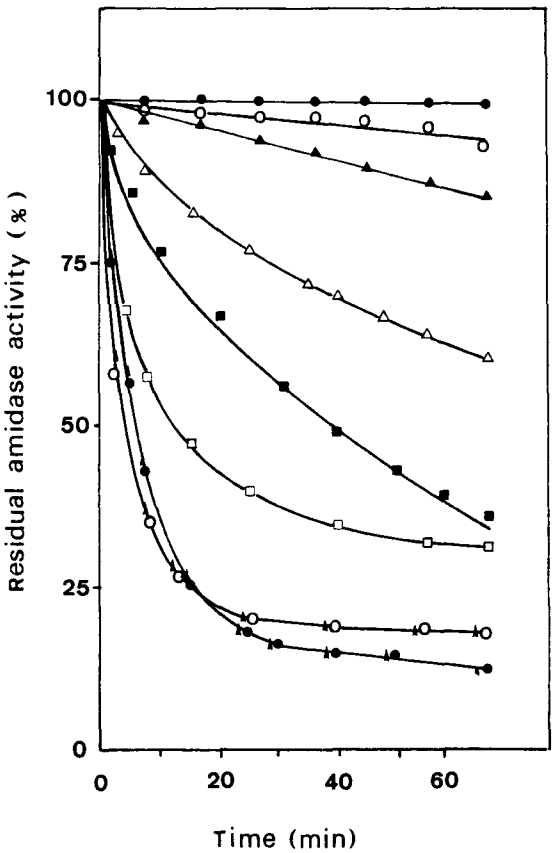

Fig. 5. Kinetics of acidic denaturation of the mPEG-PPIII conjugate (filled symbols) and of the *S*-methylthio PPIII derivative (open symbols) at 25°C. Stock solutions of PPIII were diluted in prethermostated buffers at fixed pH. (2.60, ●; 2.40, ▲; 2.21, ■; and 2.00, ▲●). At various time intervals, aliquots were removed and analyzed for amidase activity (Substrate: DL-BAPNA).

It is also observed that in marked contrast to the fluorescence of the *S*-methylthio derivative of PPIII, fluorescence of the PEG conjugate is independent of the pH, at least in the pH range from 4.0 to 9.0 examined here, as shown in Fig. 8. Interestingly, we finally observed that the fluorescence emission spectrum of the mPEG-PPIII conjugate is quite superimposable to that of reactivated PPIII measured in the acidic pH range (below pH 6.0).

The fluorescence of PPIII (*27*) like that of papain (*28*) is largely dominated by that of Trp 177 present in both enzymes. Furthermore, the fluorescence of Trp 177, which contributes to half of the total fluorescence of PPIII markedly depends on the state of protonation of the imidazole ring of His 159 in the active site of the proteinase (*27*). From the similarity between the spectra of reactivated PPIII in acidic pH range (with the imidazole ring of His 159 fully protonated) and that of the mPEG-PPIII conjugate, we can conclude that, in the conjugate, the PEG chain efficiently

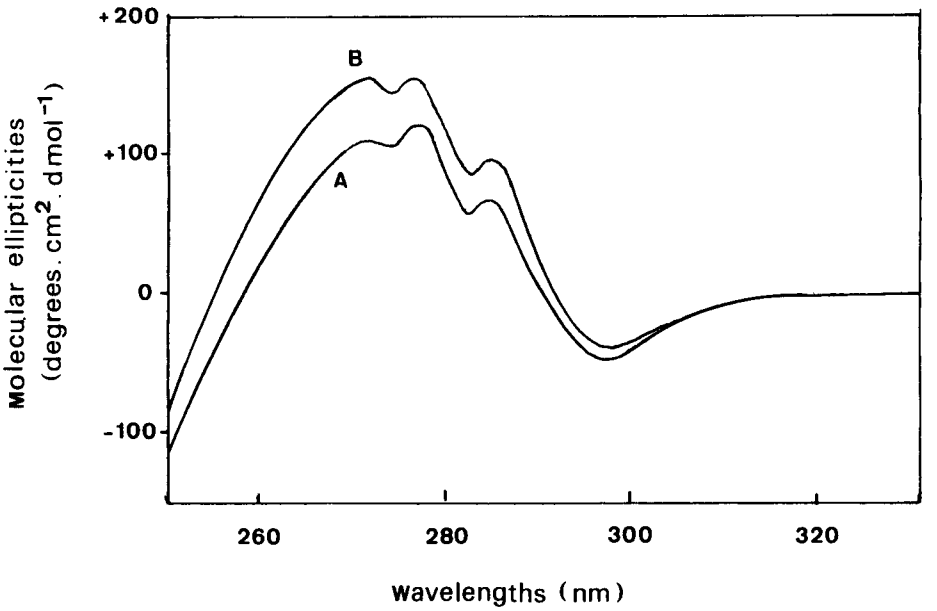

Fig. 6. Circular dichroism spectra of S-methylthio PPIII (A) and of mPEG-PPIII conjugate (B) in the near-UV range. The measurements were performed with 16 μM proteinase solutions (pH 5.0) in a 2-cm cell at 20°C.

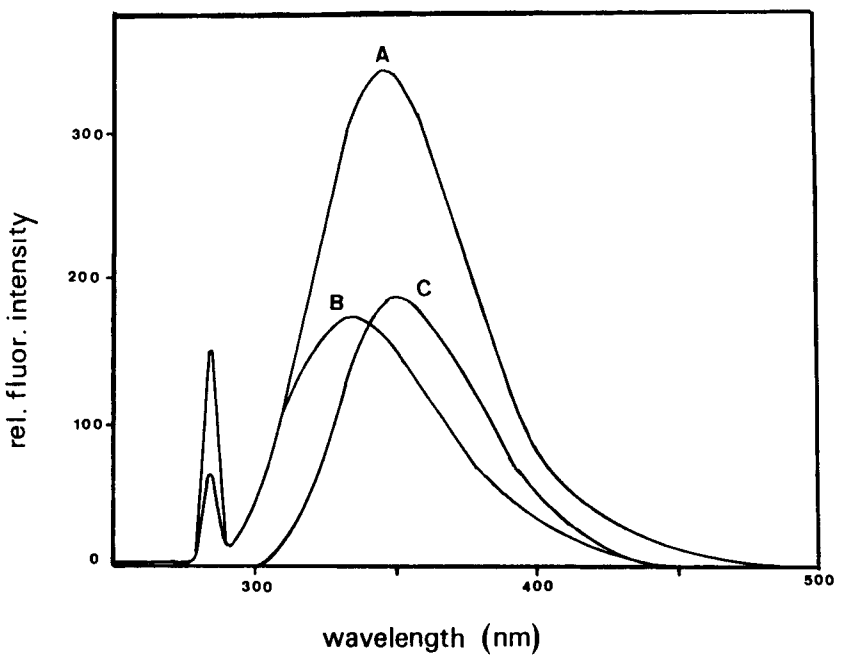

Fig. 7. Fluorescence emission spectra of the S-methylthio-PPIII (A) and of mPEG-PPIII conjugate (B). Excitation wavelength: 285 nm; excitation and emission bandwidths: 5 nm each; temperature: 25°C. PPIII solutions (4 μM) were prepared in a 50 mM Tris-HCl buffer at pH 8.0. (C) Calculated differential fluorescence emission spectra (A)-(B).

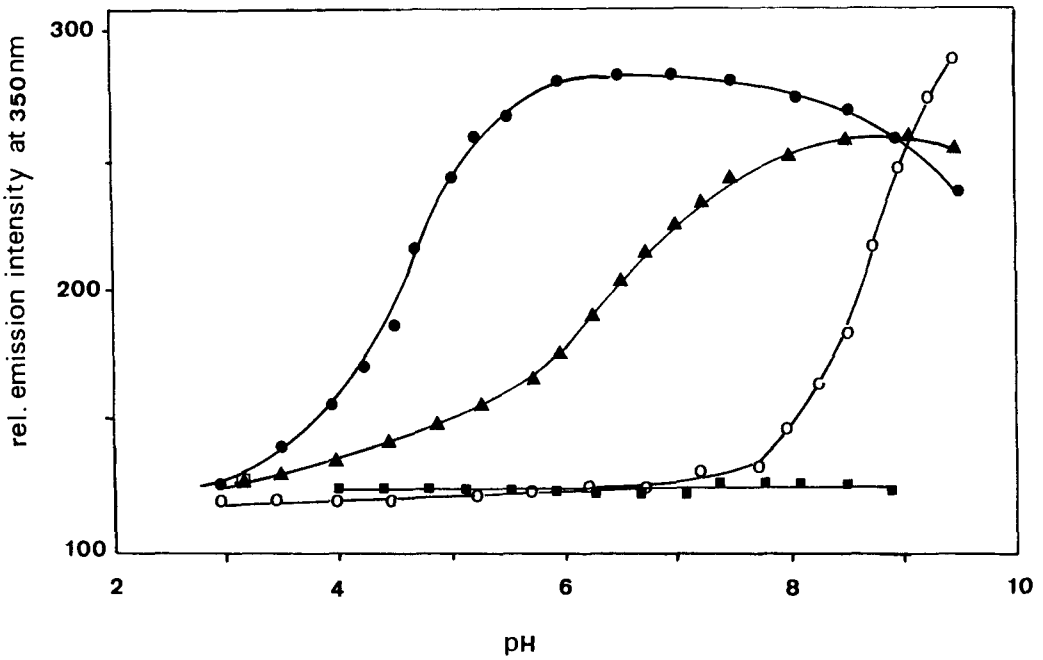

Fig. 8. Fluorescence titration, at 25°C, of *S*-methylthio PPIII (●), of reactivated PPIII (○), of irreversibly oxidized PPIII (▲), and of the mPEG–PPIII conjugate (■). For the reactivated PPIII, above pH 8.5, the fluorescence values were corrected by multiplying the observed fluorescence intensity (not shown) by the ratio of the fluorescence intensity of the *S*-methylthio PPIII in the plateau range (pH 6.0–8.5) to the fluorescence intensity of the *S*-methylthio PPIII at the highest pH of interest (*27*).

quenches the fluorescence of Trp 177. As a consequence, the emission intensity at 350 nm for the conjugate is independent of the pH as observed in Fig. 8.

In the 3-D structures of papain (*21*) and of PPIII (*14*), Trp 177 interacts with other aromatic side chains, common to both amino acid sequences (*1,29*) to form an aromatic cluster. These aromatic side chains include those of Phe 141, Tyr 144, Phe 149, and Trp 181 (papain numbering). Since the fluorescence of Trp 177 is completely quenched by the PEG chain in the mPEG–PPIII conjugate, an interaction between this chain and the above-mentioned cluster might not be excluded. It is thus expected that such an interaction might significantly affect the CD spectrum in the near-UV range of PPIII, as observed in Fig. 6. The *S*-methylthio derivative of PPIII regenerated from the mPEG–PPIII conjugate was further characterized by examining the pH dependence of its fluorescence intensity at 350 nm. As shown in Fig. 8, an apparent pK_a value of 4.50 for imidazole ring of His-159 is measured. On removal of the methylthio group, this pK_a value becomes equal to 8.60.

These values are in excellent agreement with those reported by Kaarsholm (*27*), who made use of covalent chromatography to recover a preparation of fully reactivable PPIII. The abnormally low pK_a observed for the *S*-methylthio derivatives of various cysteine-proteinases has been attributed to the hydrophobic environment of the imidazole ring of His-159 (*30*).

To test this interpretation, we examined the pH dependence of the fluorescence of our irreversibly oxidized PPIII preparation (pool 2 from Fig. 3). As shown in Fig. 8, we observe that the apparent pK_a is shifted toward a value of 6.5. By introducing an ionizable function ($-SO_2H$) in the vicinity of the imidazolyl ring, a quite normal pK_a value is restored. This result confirms the interpretation given for the low pK_a value of His-159 in the active site of these proteinases.

Extensive *N*-Acylation of the mPEG–PPIII Conjugate

In a previous work (*31*), *S*-methylthio PPIII has been extensively modified after reaction with 2 mol of mPEG-*N*-succinimide carbonate (5 kDa)/mol of amino function (*5*); 15 acylable sites on 23 were modified. The amidase activity of the conjugate (DL-BAPNA or Benzoyloxycarbonyl-Phe-Ala-Arg-7-[4-methyl]-coumaryl amide as the substrates) was, however, preserved. On the contrary, the proteolytic activity measured by using bovine serum albumin (BSA) as the substrate was completely suppressed.

It seemed thus particularly interesting to examine here to what extent the presence of the mPEG chain, anchored in the active site of PPIII, would affect the pattern of the *N*-acylation of the proteinase by mPEG-*N*-succinimide carbonate and the proteolytic activity of the PEG adduct. The mPEG–PPIII conjugate was thus acylated at 25°C and pH 7.0 (using an automatic titrator) with mPEG-*N*-succinimide carbonate (40 mol/mol of PPIII). Solid mPEG-*N*-succinimide carbonate was added in four equal portions each 20 min. Reaction was then allowed to proceed up to completion, which was indicated by the end of NaOH consumption. The reaction mixture was then dialyzed against water and analyzed for its content in free remaining amino functions. This was carried out fluorimetrically after reaction with fluorescamine (*20*). The results of this analysis are shown in Fig. 9.

From the comparison of the slopes obtained with the acylated and unacylated mPEG-N-PPIII conjugates, we can conclude that a mean value of 17 amino functions are modified. This value could have been overestimated, since some amino groups unable to react with succinic anhydride may also escape the detection by fluorescamine. An important point to underline here is the comparison of this value with the 15 amino functions of the *S*-methylthioderivative of PPIII, which were acylated using the same experimental conditions as those used here and determined using the same analytical method (*31*). One may thus conclude that the presence of the mPEG chain anchored in the active site of the proteinase does not affect the overall rate of acylation of the amino functions of PPIII.

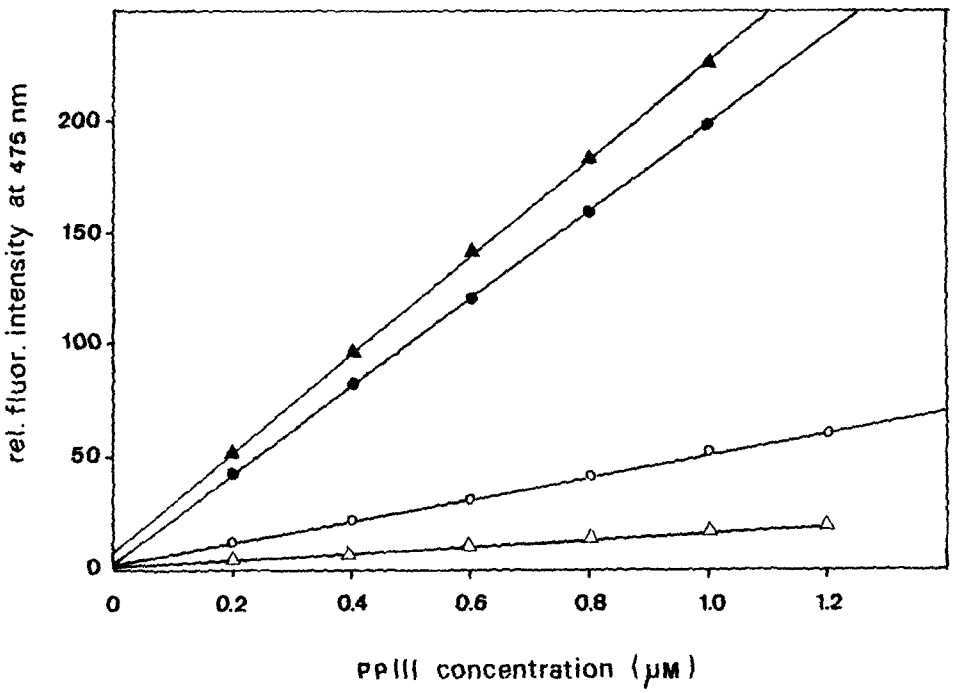

Fig. 9. Fluorimetric titration of free amino functions of irreversibly oxidized PPIII (●), of mPEG–PPIII conjugate (▲), of mPEG–PPIII conjugate after acylation with mPEG-*N*-succinimide carbonate (○), and of irreversibly oxidized PPIII after acylation with succinic anhydride (△).

However, it is expected that the pattern of *N*-acylation of the mPEG–PPIII conjugate may somewhat differ from that of *S*-methylthio PPIII. A slightly visible, but however consistently observed proteolytic activity is indeed regenerable from the fully *N*-acylated mPEG–PPIII conjugate. This is apparent in two separate series of experiments.

In the first one, azocoll (5 mg) and the fully *N*-acylated mPEG–PPIII conjugate (6 nmol) were incubated in 1 mL of a phosphate, citrate, borate (100 m*M* each) buffer at pH 6.8 in the presence of dithiothreitol (5 m*M*). Incubation at 25°C yielded soluble colored fragments from azocoll.

In another series of experiments, BSA (50 mg/mL) was incubated at 37°C in the presence of DTT (5 m*M*) with the fully *N*-acylated mPEG–PPIII conjugate (enzyme-to-substrate ratio: 4%). At various time intervals, aliquots were removed, diluted in the SDS-PAGE buffer (*see* Materials and Methods), and boiled for 4 min. The samples were then analyzed by SDS-PAGE (results not shown). Fragmentation of BSA was visible after around 30 min of incubation with the *N*-acylated mPEG–PPIII. Fragments with M_r between 33 and 60 kDa were observed.

The fully acylated PPIII is visible at the top of the gels, wherein it does not penetrate at all owing to its high molecular weight. These last results are in contrast with those observed with *N*-acetylated *S*-methylthio PPIII, where proteolytic activity could not be detected at all.

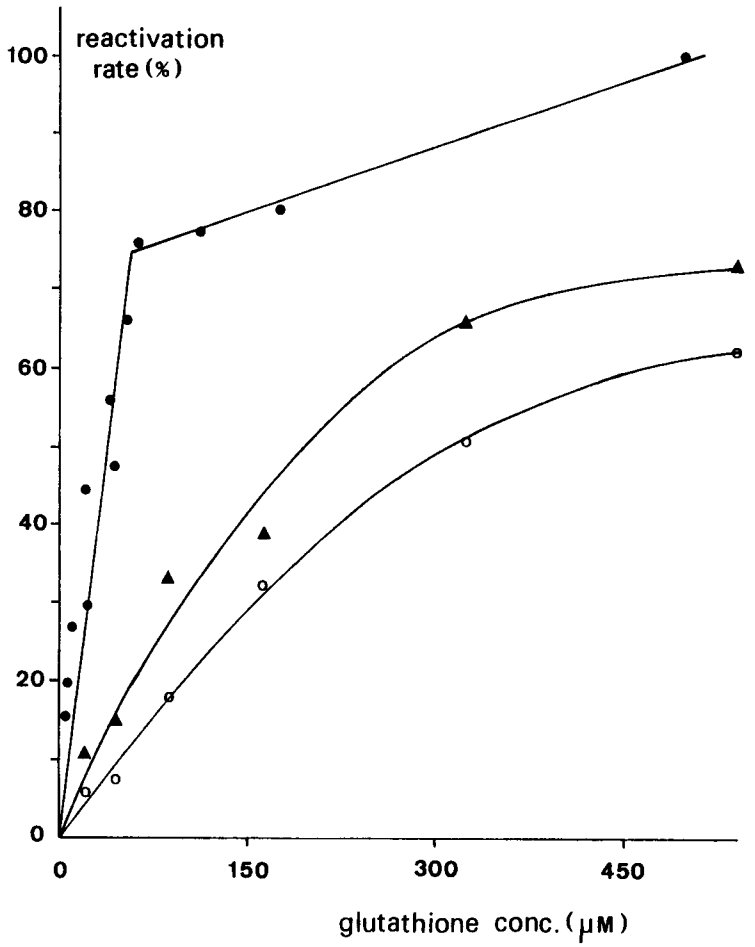

Fig. 10. Reactivation of *S*-methylthio PPIII (▲, ○) and of PEG-PPIII conjugate (●). Reactivable enzyme (2 m*M*) was incubated at 37°C for 5 (●, ○) or 15 (▲) min with glutathione in a buffer (pH 6.8) containing phosphate, citrate, and borate (100 m*M* each) in addition to EDTA (3 m*M*). Amidase activity was measured using L-BAPNA (0.5 m*M*) as the substrate. Reaction was started by adding the substrate. The release of *p*-nitroaniline from DL-BAPNA was continuously monitored at 410 nm.

Comparison of the Reactivation Rates by Glutathione

The reactivation rates of *S*-methylthio PPIII and of the mPEG-PPIII conjugate were also compared. Glutathione was used, in both cases, as the reactivating agent. Figure 10 displays the dependence, at 37°C, of the reactivation rates on the glutathione concentration. This latter was determined simultaneously using 2,2′-dipyridyl sulfide as the titrant (*11*).

Reactivation rates were defined as the percentage of amidase activity recovered under the conditions specified in Fig. 10 and compared to the amidase activity recovered after preincubating the enzyme for 10 min at 37°C, in the presence of 5 m*M* dithiothreitol.

Reactivation of the mPEG-PPIII conjugate differs from that of the *S*-methylthio PPIII according to at least two criteria. First, reactivation of the mPEG-PPIII conjugate reaches completion after 5 min, whereas 15 min are necessary for the *S*-methylthio PPIII. Furthermore, lower glutathione concentrations are needed to reactive the mPEG-PPIII conjugate fully. The mixed disulfide bond linking mPEG to PPIII looks thus particularly sensitive to thiol disulfide interchange.

CONCLUSION

Most of the work carried out in the field of covalent attachment of PEG chains to polypeptides is based on *N*-acylation reactions. The reactional mixtures obtained were generally so complex that the purification of individual molecular species was never attempted or even considered. It is obvious that from such an approach, detailed knowledge about the structural organization of PEG-polypeptide conjugates could never be obtained. Moreover, *N*-acylation leads to irreversibly modified polypeptides.

Our method, which is based on the modification of free thiol functions of proteins through the formation of disulfide bonds, takes advantage of the fact that free thiol functions in proteins are scarce and that disulfide bonds can easily be reduced under nondenaturating conditions.

In addition to the practical usefulness of our approach, it also opens prospects for a detailed study of the structure of PEG-protein conjugates. Papaya proteinase III isolated from the latex of the tropical species *Carica papaya L.* was selected for such a study, since its properties have been well investigated in our laboratory (*12,13,15*). This cysteine-proteinase contains a unique thiol function that is essential for its catalytic activity, located at position 25 in the amino acid sequence. Through the formation of a mixed disulfide bond between cysteine-25 of the enzyme and the PEG chain, we succeeded in attaching a PEG chain to PPIII and purifying to homogeneity the resulting mPEG-PPIII conjugate. This conjugate was then compared to the *S*-methylthio PPIII, another derivative of PPIII prepared from the mPEG-PPIII conjugate (*see* p. 251) and wherein the PEG moiety was substituted by a CH_3—S radical.

Several independent observations led us to conclude that the anchored PEG chain shows poor affinity for the polypeptide chain. The PEG moiety in the conjugate protects the proteinase from acidic denaturation to only a very limited extent. Moreover, most of the amino groups, including those in the vicinity of the active site in the conjugate, remain susceptible to *N*-acylation using bulky mPEG-N-succinimide carbonate. *N*-acylation of the conjugate indeed led to a PIII derivative able to regenerate little proteolytic activity (BSA as the substrate). Furthermore, one may expect that some conformational changes would result from strong interactions between the PEG and the polypeptide chains. This was not the case, as demonstrated by the FTIR spectra.

The spectral changes occurring on conjugation of PEG to the proteinase could clearly be attributed to the side chain of Trp 177 and most probably also to the four other aromatic side chains that form with Trp 177 an aromatic cluster (*14*). This means that the only observed spectral changes concern amino acid side chains located, in the 3-D structure of PPIII, close to the site of covalent attachment of the PEG chain. On the other hand, our suggestion that the PEG chain, in the conjugate, shows repulsion toward the polypeptide chain is concluded from the comparison of the reactivation rates of mPEG-PPIII conjugate and *S*-methylthio PPIII, respectively.

It was observed that active PPIII was regenerated from the mPEG-PPIII conjugate at lower glutathione concentrations and at higher rates than from the *S*-methylthio-PPIII derivative.

The properties of PEGs are those of highly flexible and highly mobile polymers (*32*). As a consequence, the covalent attachment of such polymers to proteins is unfavorable from an entropic point of view. All the observations reported here may be interpreted as an indication of the tendency of the covalently linked PEG chain to retain high flexibility and mobility.

In this perspective, the covalently linked PEG chain would recover a higher degree of mobility by reacting with glutathione. On the other hand, maintained flexibility is strengthened by the existence of repulsions toward the polypeptide chain.

In short, our results suggest that the PEG chain shows poor affinity owing to important repulsion toward the proteinase and that the repulsion force exerted by the PEG are also conferred to the mPEG-PPIII conjugate.

Repulsion forces exerted by the PEG chain toward proteins have already been exploited. For example, the reduction of fibrinogen adsorption on polystyrene surfaces was achieved by coating these surfaces with PEG (*33*).

PEGs have also been used to coat hydrophobic chromatographic supports in order to obtain ligands having a reduced hydrophobicity. This led to the concept of "mild hydrophobic chromatography" (*34–36*).

PEGs also exert repulsion forces toward other macromolecular hydrophilic substances, such as dextrans, resulting in the formation of two aqueous phase systems. This property was extensively used for the purification of proteins and/or cells (*37–41*). In the course of this work, we separated the mPEG-PPIII conjugate from the unconjugated PPIII molecular species by taking advantage of the repulsive forces exerted by PEG toward the chromatographic gels. On the other hand, repulsions toward the polypeptide moiety in the conjugate allowed us to regenerate PPIII as a fully native proteinase.

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